

5 process and release A $\beta$  peptide, levels of endogenous APP processing are low and difficult to detect by EIA. A $\beta$  processing can be increased by expressing in transformed cell lines mutations of APP that enhance A $\beta$  processing. We made the serendipitous observation that addition of two lysine residues to the carboxyl terminus of APP695 increases A $\beta$  processing  
10 still further. This allowed us to create a transformed cell line that releases A $\beta$  peptide into the culture medium at the remarkable level of 20,000 pg/ml.

#### Materials And Methods

##### Materials:

15 Human embryonic kidney cell line 293 (HEK293 cells) were obtained internally.  
20 The vector pIRES-EGFP was purchased from Clontech. Oligonucleotides for mutation using the polymerase chain reaction (PCR) were purchased from Genosys. A plasmid containing human APP695 (SEQ ID No. 9 [nucleotide] and SEQ ID No. 10 [amino acid]) was obtained from Northwestern University Medical School. This was subcloned into pSK (Stratagene) at the *Not*I site creating the plasmid pAPP695.

##### Mutagenesis protocol:

25 The Swedish mutation (K670N, M671L) was introduced into pAPP695 using the Stratagene Quick Change Mutagenesis Kit to create the plasmid pAPP695NL (SEQ ID No. 11 [nucleotide] and SEQ ID No. 12 [amino acid]). To introduce a di-lysine motif at the C-terminus of APP695, the forward primer #276 5' GACTGACCACTCGACCAGGTTC  
30 (SEQ ID No. 47) was used with the "patch" primer #274 5' CGAATTAAATTCCAGCACACTGGCTACTTCTTGTCTGCATCTCAAAGAAC (SEQ ID No. 48) and the flanking primer #275 CGAATTAAATTCCAGCACACTGGCTA (SEQ ID No. 49) to modify the 3' end of the APP695 cDNA (SEQ ID No. 15 [nucleotide] and SEQ ID No. 16 [amino acid]). This also added a BstXI restriction site that will be  
35 compatible with the BstXI site in the multiple cloning site of pIRES-EGFP. PCR amplification was performed with a Clontech HF Advantage cDNA PCR kit using the polymerase mix and buffers supplied by the manufacturer. For "patch" PCR, the patch primer was used at 1/20th the molar concentration of the flanking primers. PCR  
40 amplification products were purified using a QIAquick PCR purification kit (Qiagen).  
45 After digestion with restriction enzymes, products were separated on 0.8% agarose gels and then excised DNA fragments were purified using a QIAquick gel extraction kit (Qiagen).

50 To reassemble a modified APP695-Sw cDNA, the 5' *Not*I-BglII fragment of the APP695-Sw cDNA and the 3' BglII-BstXI APP695 cDNA fragment obtained by PCR were

5 ligated into pIRES-EGFP plasmid DNA opened at the Not I and BstX1 sites. Ligations  
were performed for 5 minutes at room temperature using a Rapid DNA Ligation kit  
(Boehringer Mannheim) and transformed into Library Efficiency DH5a Competent Cells  
(GibcoBRL-Life Technologies). Bacterial colonies were screened for inserts by PCR  
10 5 amplification using primers #276 and #275. Plasmid DNA was purified for mammalian  
cell transfection using a QIAprep Spin Miniprep kit (Qiagen). The construct obtained was  
designated pMG125.3 (APPSW-KK, SEQ ID No. 17 [nucleotide] and SEQ ID No. 18  
[amino acid]).

15 ***Mammalian Cell Transfection:***

10 HEK293 cells for transfection were grown to 80% confluence in Dulbecco's  
modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cotransfections were  
performed using LipofectAmine (Gibco-BRL) with 3 µg pMG125.3 DNA and 9 µg  
pcDNA3.1 DNA per  $10 \times 10^6$  cells. Three days posttransfection, cells were passaged into  
medium containing G418 at a concentration of 400 µg/ml. After three days growth in  
25 selective medium, cells were sorted by their fluorescence.

25 ***Clonal Selection of 125.3 cells by FACS:***

Cell samples were analyzed on an EPICS Elite ESP flow cytometer (Coulter,  
Hialeah, FL) equipped with a 488 nm excitation line supplied by an air-cooled argon laser.  
30 EGFP emission was measured through a 525 nm band-pass filter and fluorescence intensity  
was displayed on a 4-decade log scale after gating on viable cells as determined by forward  
and right angle light scatter. Single green cells were separated into each well of one 96 well  
plate containing growth medium without G418. After a four day recovery period, G418  
35 was added to the medium to a final concentration of 400 µg/ml. After selection, 32% of the  
wells contained expanding clones. Wells with clones were expanded from the 96 well plate  
to a 24 well plate and then a 6 well plate with the fastest growing colonies chosen for  
expansion at each passage. The final cell line selected was the fastest growing of the final  
40 six passages. This clone, designated 125.3, has been maintained in G418 at 400 µg/ml with  
passage every four days into fresh medium. No loss of Aβ production of EGFP  
fluorescence has been seen over 23 passages.

45 30 ***Aβ EIA Analysis (Double Antibody Sandwich ELISA for hAβ 1-40/42):***

Cell culture supernatants harvested 48 hr after transfection were analyzed in a  
standard Aβ EIA as follows. Human Aβ 1-40 or 1-42 was measured using monoclonal  
50 antibody (mAb) 6E10 (Senetek, St. Louis, MO) and biotinylated rabbit antiserum 162 or

164 (New York State Institute for Basic Research, Staten Island, NY) in a double antibody sandwich ELISA. The capture antibody 6E10 is specific to an epitope present on the N-terminal amino acid residues 1-16 of hA $\beta$ . The conjugated detecting antibodies 162 and 164 are specific for hA $\beta$  1-40 and 1-42, respectively. Briefly, a Nunc Maxisorp 96 well immunoplate was coated with 100  $\mu$ l/well of mAb 6E10 (5  $\mu$ g/ml) diluted in 0.1M carbonate-bicarbonate buffer, pH 9.6 and incubated at 4°C overnight. After washing the plate 3x with 0.01M DPBS (Modified Dulbecco's Phosphate Buffered Saline (0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01 M potassium chloride, pH 7.4) from Pierce, Rockford, IL) containing 0.05% of Tween-20 (DPBST), the plate was blocked for 60 min with 200  $\mu$ l of 10% normal sheep serum (Sigma) in 0.01M DPBS to avoid non-specific binding. Human A $\beta$  1-40 or 1-42 standards 100  $\mu$ l/well (Bachem, Torrance, CA) diluted, from a 1 mg/ml stock solution in DMSO, in culture medium was added after washing the plate, as well as 100  $\mu$ l/well of sample, e.g. conditioned medium of transfected cells. The plate was incubated for 2 hours at room temperature and 4°C overnight. The next day, after washing the plate, 100  $\mu$ l/well biotinylated rabbit antiserum 162 1:400 or 164 1:50 diluted in DPBST + 0.5% BSA was added and incubated at room temperature for 1 hr 15 min. Following washes, 100  $\mu$ l/well neutravidin-horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in DPBST was applied and incubated for 1 hr at room temperature. After the last washes 100  $\mu$ l/well of o-phenylenediamine dihydrochloride (Sigma Chemicals, St. Louis, MO) in 50mM citric acid/100mM sodium phosphate buffer (Sigma Chemicals, St. Louis, MO), pH 5.0, was added as substrate and the color development was monitored at 450nm in a kinetic microplate reader for 20 min. using Soft max Pro software. All standards and samples were run in triplicates. The samples with absorbance values falling within the standard curve were extrapolated from the standard curves using Soft max Pro software and expressed in pg/ml culture medium.

#### Results:

Addition of two lysine residues to the carboxyl terminus of APP695 greatly increases A $\beta$  processing in HEK293 cells as shown by transient expression (Table 1). Addition of the di-lysine motif to APP695 increases A $\beta$  processing to that seen with the APP695 containing the Swedish mutation. Combining the di-lysine motif with the Swedish mutation further increases processing by an additional 2.8 fold.